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Inhibins, activins and follistatin in reproduction

D.M.de Kretser[1], M.P.Hedger, K.L.Loveland and D.J.Phillips

Centre for Molecular Reproduction and Endocrinology, Monash Institute of Reproduction and Development, Monash University, Clayton 3168, Victoria, Australia

[1]To whom correspondence should be addressed at: Monash Institute of Reproduction and Development, Monash Medical Centre, 246 Clayton Road, Clayton 3168, Victoria, Australia; E-mail: david.de.kretser@med.monash.edu.au

The regulation of reproductive processes involves a complex network of communication systems between the brain, endocrine organs, the gonads and other reproductive tissues. Classically, our understanding has focused on the role of endocrine hormones, but more recently interest has also dwelt on the paracrine and autocrine regulation of these cell systems. In this review, the structure and physiology of the inhibins, activins and follistatin are discussed in terms of the evidence supporting their role as endocrine hormones, and how they might function as paracrine factors within the pituitary, gonad and associated tissues. With the advent of more specific techniques and assays for their measurement, the potential of inhibins, activins and follistatin as clinical markers of reproductive function and in the screening of various pathologies is also evaluated.

Keywords: endocrine/FSH/ovary/paracrine/testis

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Introduction

The quest to isolate inhibin, a putative protein hormone produced by the testis and ovary, culminated in 1985 with the purification of two forms of inhibin, namely inhibin A and inhibin B (Ling *et al.*, 1985; Robertson *et al.*, 1985). These proteins were shown to be disulphide-linked dimers which shared a common α-subunit and differed on the basis of a β-subunit termed β_A in inhibin A ($\alpha\beta_A$) and β_B in inhibin B ($\alpha\beta_B$). Both inhibin A and inhibin B have the capacity specifically to suppress FSH secretion by pituitary cells in culture, without affecting LH secretion.

In subsequent years, our understanding of the control of FSH has increased in complexity following the isolation from follicular fluid of three proteins termed activin A, activin B and activin AB, all of which could stimulate FSH secretion (Ling *et al.*, 1986; Vale *et al.*, 1986). Each of the activins represent disulphide-linked dimers of the β-subunits of inhibin, either homodimers of the β-subunit (activin A: $\beta_A\beta_A$, activin B: $\beta_B\beta_B$) or a heterodimer (activin AB: $\beta_A\beta_B$). The β_A, β_B and α-subunits show homology to each other and also are members of the transforming growth factor-β (TGFβ) superfamily of proteins (Kingsley, 1994). Subsequently, three additional β-subunits termed β_C, β_D (*Xenopus* only) and β_E have been isolated with disulphide-linked homodimers being formed to create activin C, activin D and activin E (Hötten *et al.*, 1995; Oda *et al.*, 1995; Fang *et al.*, 1996). These dimers have no effect on FSH secretion, but it is important to note that the β_C-subunit has the capacity to form heterodimers with β_A and β_B but not with the α-subunit (Mellor *et al.*, 2000). Thus, synthesis of the β_C-subunit may affect the levels of bioavailable activin.

Further studies of proteins in ovarian follicular fluid enabled the isolation of another FSH-suppressing protein termed follistatin, that bore no significant homology to the α- and β-subunits of the inhibin/activin family (Robertson *et al.*, 1987; Ueno *et al.*, 1987). Follistatin is a single-chain glycoprotein hormone with a range of molecular weights from 31 to 49 kDa based on alternative mRNA splicing and variable glycosylation of the protein. The alternatively spliced mRNAs encode two proteins of 315 amino acids (follistatin 315) and 288 amino acids (follistatin 288); follistatin 315 can be further proteolytically degraded to follistatin 303. Both follistatin 315 and follistatin 288 have the capacity to suppress FSH specifically *in vitro* and *in vivo*. Subsequent studies have clearly identified that the mechanism of action of follistatin on FSH secretion results from its capacity to bind activin with high affinity, thereby neutralizing the FSH stimulatory actions of activin (Nakamura *et al.*, 1990). It is unclear whether follistatin has an action independent of its capacity to bind and neutralize the function of activins A, AB and B.

As will be discussed in more detail below, the overwhelming view is that inhibin acts as a circulating feedback regulator of FSH secretion. Although it is produced at a number of sites, the ovary and testis form the major circulating sources of inhibin (Robertson *et al.*, 1988). In contrast, the activins are produced in a much broader range of tissues (Meunier *et al.*, 1988) and have a vast array of actions usually exerted through paracrine mechanisms (Woodruff, 1998). Whereas gonadectomy leads to a precipitous and rapid decline in circulating inhibin concentrations (Robertson *et al.*, 1988; Ishida *et al.*, 1990), similar experiments did not result in dramatically lower levels of activin and follistatin concentrations (Sakai *et al.*, 1992; Klein *et al.*, 1993; McFarlane *et al.*, 1996; Phillips *et al.*, 1996), strongly suggesting the presence of multiple sources of these proteins. In fact, the levels of follistatin rise following castration (Phillips *et al.*, 1996); this latter response represents part of the acute phase response following surgical stress.

The biology of follistatin leads to the existence of reservoirs of activin and follistatin in a variety of tissue sites. Follistatin 288 binds to heparan sulphate proteoglycans (HSPG) (Sugino *et al.*, 1993) and consequently, by its ability to bind activin, significant stores of follistatin 288 and activin are found complexed to HSPG in basement membranes. This concept has been confirmed by the demonstration that heparin, when infused into the circulation, has the capacity to stimulate large and rapid releases of follistatin and activin (Klein *et al.*, 1996a; Phillips *et al.*, 2000). The biological importance of the released follistatin and activin remains unclear, especially in view of studies suggesting that the binding of activin to follistatin targets activin to a lysosomal intracellular degradation pathway (Hashimoto *et al.*, 1997). Unlike the binding of fibroblast growth factors (FGF) to HSPG, which enhances the accessibility of FGF to their receptors (McKeehan *et al.*, 1998), no such augmented action results from the binding of activin to follistatin bound to HSPG. In contrast, follistatin 315 has a low affinity for HSPG and is regarded as the major circulating form of follistatin (Schneyer *et al.*, 1996).

Follistatin is often produced in the same cells that produce activin, or alternatively in adjacent cell types. These sites of production serve to regulate the local actions of activin and perhaps, through concentration gradients, limit the capacity of this multi-potent growth factor to diffuse into the circulation and exert actions at distant tissues (Phillips and de Kretser, 1998; Phillips, 2000).

Impact of inhibin, activin and follistatin on reproductive processes by regulation of FSH secretion

As briefly mentioned above, there is agreement that inhibin is principally produced in the testis and ovary and, by circulating in the blood stream, exerts its action at the pituitary to suppress FSH secretion. The principal sites of production in the male are the Sertoli cells (Steinberger and Steinberger, 1976; Roberts *et al.*, 1989; Anawalt *et al.*, 1996; Majdic *et al.*, 1997), and in the female, the granulosa cells (Findlay *et al.*, 2001). Infusion of recombinant human inhibin A into the circulation in castrated rams resulted in a specific suppression of FSH secretion commencing approximately 6 h after the start of the infusion and continuing for a period of approximately 12 h following its cessation (Tilbrook *et al.*, 1993). While the effects of inhibin on gonadotrophin secretion are directed exclusively at the pituitary and for FSH are independent of GnRH inputs, there are some suggestions that inhibin may decrease LH pulse amplitude under conditions of low GnRH (Tilbrook *et al.*, 2001). The responsiveness of pituitary FSH to inhibin feedback is set up early in postnatal life, with the maximum sensitivity occurring by the age of puberty (Tilbrook *et al.*, 1999).

In contrast to the actions of inhibin, which typify a circulating negative feedback regulator, the activins exert their action on FSH secretion through paracrine mechanisms in the pituitary (Corrigan *et al.*, 1991). However, infusions of activin A have been shown to stimulate FSH secretion in monkeys (McLachlan *et al.*, 1989; Stouffer *et al.*, 1993). The activin β_B and β_A subunits are produced by gonadotrophs in the pituitary and have been shown to act locally to exert a stimulatory action on FSH secretion (Roberts *et al.*, 1989). Exposure of pituitary cells in culture to a neutralizing monoclonal antibody to activin B resulted in a decline in FSH secretion *in vitro* (Corrigan *et al.*, 1991; Bilezikjian *et al.*, 1993a,b, 1996). Similarly, exposure of the pituitary to follistatin, in the presence or absence of exogenous activin, can block activin's stimulatory actions on FSH. Several studies have demonstrated that follistatin is also produced in the pituitary glands in the folliculo-stellate cells and can modulate the local actions of activin on FSH secretion (Gospodarowicz and Lau, 1989; Kogawa *et al.*, 1991).

Role of inhibins, activins and follistatin in the male

Actions in the testis

In order to understand fully the actions of these proteins in the testis, it is crucial to review the distribution of the α, β_A and β_B inhibin subunits, follistatin, and their receptors and binding sites within the various cell types. Their localization to the testis has been confirmed by the identification of mRNA and protein at several sites, and these will be discussed below and related to our understanding of their functional significance.

As indicated earlier, the testis is a major contributor of circulating inhibin levels. In the human, inhibin B is the circulating form whereas in the sheep it is inhibin A (Illingworth *et al.*, 1996a). Castration leads to a profound and rapid fall in circulating inhibin levels (Robertson *et al.*, 1988). The levels of inhibin in testicular venous blood were higher than levels in the general circulation, but the gradient was considerably lower than that seen for steroid hormones (Ishida *et al.*, 1990; Winters, 1990). These observations are consistent with a longer half-life for inhibin compared with testosterone. Immunohistochemistry and in-situ hybridization data demonstrate that production of the inhibin α-subunit mRNA and protein within the seminiferous epithelium occurs within Sertoli cells but not germ cells, and within Leydig cells of the immature and adult testis (Cuevas *et al.*, 1987; Shaha *et al.*, 1989; Kaipia *et al.*, 1992; Majdic *et al.*, 1997; Noguchi *et al.*, 1997).

There are considerable data available to indicate that Sertoli cells are the principal site of inhibin production in the male, as shown by the secretion of inhibin *in vitro* by Sertoli cell cultures and its stimulation by FSH (Steinberger and Steinberger, 1976; Le Gac and de Kretser, 1982). There is also a positive relationship between Sertoli cell number and inhibin B levels in the circulation

in a number of normal and pathophysiological states in the rat and monkey, thereby supporting the concept that the Sertoli cell is the principal site of inhibin B production (Ramaswamy *et al.*, 1999; Sharpe *et al.*, 1999).

FSH is generally regarded as the principal stimulatory protein for inhibin secretion, acting to up-regulate α-subunit mRNA production (Steinberger and Steinberger, 1976; Le Gac and de Kretser, 1982; Hancock *et al.*, 1992). Application of high doses of FSH to rat Sertoli cells *in vitro* caused the secretion of both dimeric inhibin and free α-subunit precursors (Grootenhuis *et al.*, 1989; Hancock *et al.*, 1992). These observations suggest that Sertoli cells not only secrete dimeric inhibin but may also contribute free α-subunit products into the circulation. There is also evidence that the Leydig cells have the capacity to produce both bioactive and immunoactive inhibin in humans and rats (McLachlan *et al.*, 1988; Risbridger *et al.*, 1989). However, the stimulation of circulating immunoactive inhibin B levels by hCG in humans has not been confirmed using dimeric enzyme-linked immunosorbent assays (ELISAs) (Kinniburgh and Anderson, 2001), which detected a rapid rise of the α-subunit precursor, pro-α_C. Recent studies using the Leydig cell cytotoxin, ethane dimethane disulphonate (EDS), suggest that the Leydig cells, through mechanisms which are not fully understood, can modulate expression of the inhibin α-subunit gene in the seminiferous tubules (Tena-Sempere *et al.*, 1999).

As indicated earlier, there is no marked decline in activin A and follistatin levels following castration (McFarlane *et al.*, 1996; Phillips *et al.*, 1996). While this suggests that the testis does not contribute significantly to circulating levels of these two proteins, there are nevertheless data to indicate that mRNA and protein for the β_A and β_B subunits are present in Sertoli cells of the rat (Toboesch *et al.*, 1988; Kaipia *et al.*, 1992; Majdic *et al.*, 1997) and human (Andersson *et al.*, 1998; Anderson *et al.*, 1998). Studies by others (Grootenhuis *et al.*, 1989; de Winter *et al.*, 1993) have indicated the presence of activin-like bioactivity in low concentrations in Sertoli cell culture medium, and our own data using specific ELISA assays for activin A clearly demonstrate that Sertoli cells from immature and adult rats can produce this protein *in vitro* (Okuma *et al.*, 2000). There are also data indicating that the Leydig cells and peritubular cells secrete activin A *in vitro* (Lee *et al.*, 1989; de Winter *et al.*, 1994). Immunocytochemical studies in the human testis have identified β_B protein in spermatogonia, primary spermatocytes and round spermatids, raising the possibility that these are sites of activin B production (Andersson *et al.*, 1998). Similarly, our unpublished data in the rat show that the identical germ cells produce mRNA and protein for the β_B-subunit (M.K.O'Bryan *et al.*, unpublished observations).

In defining the possibility of autocrine or paracrine actions within the testis, the localization of activin receptors assists in delineating possible target tissues. mRNA for the type IIA activin receptors is located in primary spermatocytes and early round spermatids (de Winter *et al.*, 1992; Kaipia *et al.*, 1992), whilst spermatogonia express mRNA for the activin type IIB receptor (Kaipia *et al.*, 1993). Leydig and Sertoli cells also express mRNA for the type IIA receptor (de Winter *et al.*, 1992). Recent studies suggest a temporal up-regulation of a 6 kb transcript of the type IIA receptor in rat Sertoli cells at a time when they demonstrate a proliferative response to activin A, whereas the mRNA for the type IIA receptor was widely expressed (Fragale *et al.*, 2001). Expression of mRNA for the type IA receptor was found in Leydig and Sertoli cells but not in germ cells, whereas the mRNA for the ActR IB was found in germ cells, especially round spermatids (de Jong, 1997).

The functionality of these receptors has also been demonstrated by the binding of iodinated activin A to primary spermatocytes and round spermatids (Krummen *et al.*, 1994), strongly suggesting that these are sites of activin action. Since follistatin has been shown to be produced by the Sertoli cells, spermatogonia, primary spermatocytes and round spermatids, these cells have the capacity, through follistatin, to modulate the local actions of activin (Michel *et al.*, 1990; Kogawa *et al.*, 1991; Meinhardt *et al.*, 1998). As discussed earlier, the use of certain antibodies to follistatin demonstrate that this protein can be located on the outer surface of cells within the testis, suggesting binding with strong affinity to HSPG (Sugino *et al.*, 1993).

Evidence of local actions emerging from manipulation of the α, β_A and β_B genes

Matzuk *et al.* (1992) demonstrated that targeted disruption of the inhibin α-subunit gene in mice resulted in the development of testicular tumours at 3–4 weeks after birth, leading to progressive cachexia and death. Histologically, these tumour cells appeared to be very similar to granulosa cells, although they probably arise from Sertoli cells. The cachexia in these animals has been demonstrated to be due to the high levels of activin that are present in these mice since the absence of α-subunit leads to β-subunit dimerization (Matzuk *et al.*, 1994; Coerver *et al.*, 1996). By crossing these mice with α-subunit deletions with mice having a targeted disruption of the activin Type IIA receptor, it could be demonstrated that in the absence of activin signalling through this receptor, gonadal tumours still occurred but the cachexia was absent (Coerver *et al.*, 1996). This infers the importance of activin in the development of the cachexia in these mice.

Targeted disruption of the β_A-subunit gene led to perinatal death of mice due to palatal developmental defects (Matzuk *et al.*, 1995a), making it impossible to determine the effect of the absence of β_A protein on testicular development and function. In other studies, no obvious disruption of spermatogenesis was observed in mice with a functional disruption of the β_B-subunit gene (Vassalli *et al.*, 1994). It is possible that the failure to obtain a phenotype may have been due to the capacity of activin A to substitute for activin B. In a further attempt to disrupt the action of the activins, one group (Matzuk *et al.*, 1995b) showed that mice with a targeted disruption of the activin Type IIA receptor gene were fertile despite having testes that were considerably smaller than normal. These mice had lower FSH levels, and the smaller testicular size was attributed to the decrease in Sertoli cell numbers that resulted from the reduced drive by FSH on the proliferation of these cells in the fetal and neonatal periods. Quantitative studies demonstrated a 30% decrease in final Sertoli cell numbers leading to a corresponding decline in sperm production. These findings were similar to observations of mice with targeted disruption of the FSH β-subunit gene (Wreford *et al.*, 2001). Further studies in both these animal models have suggested that, in the absence of FSH, the capacity of Sertoli cells to support germ cells is decreased (Kumar *et al.*, 2001).

Other studies utilizing overexpression of the β_A-subunit gene in mice showed spermatogenic disruption (Tanimoto *et al.*, 1999). Induction of β_A synthesis in spermatocytes using the metallothionein promoter led to a heterogeneous appearance in testicular histology. In some tubules, vacuolation suggested the total absence of germ cells, but in other areas spermatids were more severely depleted than spermatogonia and primary spermatocytes. Surprisingly, overexpression of the follistatin gene also resulted in spermatogenic defects and infertility related to the degree of follistatin overexpression (Guo *et al.*, 1998). The higher the level of follistatin expression, the lower the testis weight and the greater the disruption of spermatogenesis. Unfortunately, targeted disruption of the follistatin gene leads to perinatal death due to respiratory difficulties, and hence the effects of the absence of this gene on adult testicular function cannot be studied at present (Matzuk *et al.*, 1995c). These data infer that graded levels of activin bioactivity may be crucial for determining the progress of spermatogenesis. Further support for this concept was gained when transgenic mice were generated with a construct that directed expression of the β_B-subunit coding region into the β_A-subunit gene locus (Brown *et al.*, 2000). These mice have a delay in the onset of spermatogenesis, presumably arising from the production of the less potent activin B (Corrigan *et al.*, 1991; Nakamura *et al.*, 1992) at sites where activin A would normally be made.

Actions on the development of the rodent testis

Several studies have provided data to suggest that activin and follistatin exert discrete, age-specific actions during the early post-natal development of the rat testis. This is supported by the demonstration of discrete switches in the expression of mRNAs and proteins corresponding to the receptor, ligand and antagonists involved in activin signalling. For example, mRNA and protein corresponding to the β_A-subunit are synthesized in gonocytes but they are absent from the emerging spermatogonial population that arises in the first few days of life (Meehan *et al.*, 2000). In contrast, follistatin mRNA is absent from gonocytes at birth and appears in these germ cells around day 3 as they transform into spermatogonia. Follistatin mRNA and protein persist in spermatogonia through adulthood (Meinhardt *et al.*, 1998; Meehan *et al.*, 2000). Localization of activin A to fetal gonocytes has also been demonstrated in sheep (Jarred *et al.*, 1999).

Experimental manipulations *in vitro* using testis fragment cultures have also demonstrated age-specific effects of activin. Cultures of 3-day-old rat testis resulted in activin A stimulating gonocyte numbers without an alteration in spermatogonial numbers (Meehan *et al.*, 2000). However, follistatin in combination with FSH stimulated spermatogonial numbers. In testis fragments taken from 3-day-old rats, activin A either slightly inhibited Sertoli cell proliferation (Meehan *et al.*, 2000) or had no measurable effect (Boitani *et al.*, 1995), while FSH strongly stimulated Sertoli cell proliferation. In contrast, in fragments from day 9 rats, a stimulation of Sertoli cell proliferation was observed, particularly by the combination of activin A and FSH (Boitani *et al.*, 1995). A discrete up-regulation of the 6 kb mRNA isoform encoding the activin type II receptor was documented in Sertoli cells of the 7- to 9-day-old rat (Fragale *et al.*, 2001), corresponding to the time when activin has a stimulatory effect on Sertoli cell proliferation and immediately prior to the onset of Sertoli cell terminal differentiation. These temporally discrete actions suggest important regulatory roles for these proteins during early testicular development. In co-cultures of spermatogonia with Sertoli cells from day 20 rats, a significant stimulation of spermatogonial proliferation in response to activin A was demonstrated (Mather *et al.*, 1990). The different results obtained in the earlier study of Mather and colleagues compared with those in the later studies using testis fragment cultures (Meehan *et al.*, 2000) may reflect the impact of the testis cytoarchitecture on activin and FSH signalling, or arise from age-specific differences in the spermatogonial populations investigated

Action on mitochondrial morphology in primary spermatocytes

During spermatogenesis, the mitochondria in spermatogonia show features similar to those in mitochondria present in somatic cells, namely that the cristae run transversely across the mitochondrial matrix. As spermatogonia enter meiosis and become primary spermatocytes, there is a dilatation of the intra-cristal spaces and the membranes of the cristae are pushed towards the periphery of the mitochondria, leaving a central space. These appearances are referred to as a 'condensed' mitochondrial morphology (Seitz *et al.*, 1995). If primary spermatocytes are cultured, there is a decrease in the percentage of mitochondria showing the condensed appearance (Meinhardt *et al.*, 2000). These authors showed that addition of Sertoli cell culture medium or activin A could, in a dose-dependent fashion, maintain the 'condensed' mitochondrial phenotype. These changes were inhibited by an antiserum specific to activin A.

The functional importance of these structural changes remains unclear. Other studies have shown that the change in mitochondrial morphology is associated with a loss of heat-shock protein 60 (HSP60) which appears to act in concert with HSP10 as a chaperone to fold newly formed mitochondrial proteins (Meinhardt *et al.*, 1995; Paranko *et al.*, 1996). Further, the 'Lon-protease' found to be localized in mitochondria was lost after the zygotene stage (Seitz *et al.*, 1995). This protease is thought to permit limited proteolytic degradation of mitochondrial proteins in the early meiotic stages, and its disappearance may indicate the loss of proteolytic degradative capacity. Given that mRNA for the β_B-subunit and its protein is localized to primary spermatocytes (Andersson *et al.*, 1998) and that these cells also express specific activin Type I and Type II receptors, the possibility exists that these actions on mitochondria may represent an example of an autocrine role for the activins.

Actions on androgen production

A series of initial observations (Hsueh *et al.*, 1987) suggested that inhibin stimulated testosterone production during in-vitro incubations of Leydig cells from 21-day-old rats, but activin had the opposite action. Others have been unable to show that inhibin stimulated testosterone production using highly purified adult Leydig cells (Lin *et al.*, 1989), suggesting therefore that this stimulation may be related to an age-specific role. On the other hand, the latter study demonstrated that activin A could, *in vitro*, inhibit testosterone production. The in-vivo functional significance of these observations remains to be determined.

Actions on the prostate

All the inhibin subunits are expressed in the prostate, and their identification was initially made in studies of the rat (Risbridger *et al.*, 1996; Ying *et al.*, 1997). Within non-malignant areas in biopsies of human prostate tissue taken from men with benign prostate hyperplasia, the inhibin subunits have been identified at both the protein and mRNA level (Thomas *et al.* 1998). The α- and β_A-subunits are localized to the basal and secretory epithelial cells in the acini, but the β_B-subunit is found principally in the basal cells and the stromal smooth muscle cells. Follistatin is principally localized to the stromal smooth muscle cells in the normal prostate.

In biopsies from men with high-grade prostate cancer, expression of the β-subunits was similar to that in normal subjects, but the α-subunit appeared to be decreased in the epithelium (Thomas *et al.*, 1997). Follistatin was present within the stroma but some cancer cells also expressed this protein. Expression of the α-subunit could not be demonstrated in the prostate cancer cell lines, LNCaP, DU145 and PC3, but these cells contained both β-subunits. Functional studies on both primary prostate epithelial cell lines and the LNCaP cell line showed that activin A could inhibit the proliferation of both basal and androgen-stimulated proliferation and induced apoptosis (Dalkin *et al.*, 1996; Wang *et al.*, 1996, 1999; McPherson *et al.*, 1997; Zhang *et al.*, 1997). In contrast, activin A could not produce a similar inhibition of proliferation when applied to PC3 cells (Dalkin *et al.*, 1996; McPherson *et al.*, 1997). In this context, both the LNCaP and PC3 cell lines express the secreted form of follistatin—follistatin 315—but only the PC3 cells expressed the membrane-bound form—follistatin 288 (McPherson *et al.*, 1999). The possibility that the activin 'resistance' was due to the expression of follistatin 288 was confirmed by the neutralization of follistatin 288 using an antiserum and subsequent restoration of the inhibitory action of exogenous activin A.

Potential clinical applications

Circulating marker of Sertoli cells

Investigators involved in the evaluation of infertile males have long sought a marker of Sertoli cell function that could be measured in blood. Several studies in rats and primates have demonstrated that serum levels of inhibin B are directly related to the number of Sertoli cells in the testis (Ramaswamy *et al.*, 1999; Sharpe *et al.*, 1999). Moreover, since FSH stimulates inhibin secretion, the levels in serum also reflect the degree of FSH stimulation and the capacity of the Sertoli cells to respond. Further data are required to confirm the value of these suggestions, but they may be useful for example in identifying patients who would benefit from further FSH stimulation in order to raise sperm counts (Foresta *et al.*, 1999).

Marker of prostate cancer

A recent review (Risbridger *et al.*, 2001) has summarized the available information concerning the distribution and actions of the inhibin-related proteins in prostate cancer. Further studies are required to confirm the concept which suggests that the distribution and levels of these proteins may provide prognostic data regarding prostate cancer.

Role of inhibins, activins and follistatin in the female

At present, our knowledge of the role of the inhibins, activins and follistatins in female reproduction is more substantial than in the male. In part, this arises from the ability to study certain aspects of ovarian folliculogenesis *in vitro*, whereas the capacity to study the seminiferous epithelium *in vitro* is compromised by the inability to maintain the integrity of the epithelium in culture.

Many of the early physiological studies of inhibin in the human menstrual cycle were undertaken by a radioimmunoassay ('Monash' assay) that was subsequently shown to measure not only the dimeric inhibins but also products of the α-subunit. This cross-reactivity resulted in an inability to distinguish between inhibin A and inhibin B and α-subunit proteins which were abundantly released into the circulation. This has required some revision of the conclusions reached from these early studies. Using this assay system, a modest increase in immunoactive inhibin was found in the late follicular phase of the menstrual cycle reaching a peak with the LH surge (McLachlan *et al.*, 1987a). The highest levels were found, surprisingly, in the luteal phase of the cycle with a positive correlation to progesterone levels. Further support for the concept that the corpus luteum secreted inhibin emerged from the ability of hCG to prevent the premenstrual decline in serum inhibin levels and the ability for hCG to prevent the dramatic decrease in the luteal phase inhibin levels following the administration of GnRH antagonist (Roseff *et al.*, 1989). These conclusions were supported by studies involving lutectomy (Illingworth *et al.*, 1991).

The availability of sensitive ELISAs for inhibin A and inhibin B enabled definition of the patterns of secretion of these two proteins across the menstrual cycle (Groome *et al.*, 1994, 1996; Muttukrishna *et al.*, 1994). These studies showed that the pattern of secretion of inhibin A very closely resembled the menstrual profile of immunoactive inhibin as measured by radioimmunoassay. In contrast, inhibin B levels are highest in the early to mid-follicular phase, and decline in the late follicular phase, rising briefly in concert with the LH surge and declining to a nadir approximately 7 days post-ovulation. This latter observation indicates that the corpus luteum does not produce inhibin B.

Since both inhibin A and inhibin B have the capacity to suppress FSH secretion, the combined actions of these proteins, in concert with estradiol, are responsible for the feedback regulation of FSH secretion from the pituitary gland. The determination of the relative potency of inhibin A and B has been difficult due to a variety of bioassays and immunologically based assays, but one study (Robertson *et al.*, 1996) represents the most thorough to date. As the levels of inhibin A and estradiol run similar courses, it is difficult to delineate the specific role for each in the control of FSH in the latter part of the follicular phase. However, the rise of inhibin B in the early to mid-follicular phase is responsible for the decline in FSH levels in the latter part of the follicular phase.

These patterns of inhibin A and inhibin B reflect the localization and production of these two proteins in the various stages of follicular development (Roberts *et al.*, 1993). The pre-antral follicle shows evidence of β_B production with no α-subunit being detected, suggesting that the dimeric product is activin B. With follicular growth, FSH stimulates α-subunit production which, together with the β_B-subunit, is responsible for inhibin B production. Further support for the view that inhibin B is

produced by these small follicles is suggested by the rise in FSH levels in perimenopausal women, brought about in part by the declining ovarian output of inhibin B from the reduced number of small follicles (Welt *et al.*, 1999).

As the cohort of follicles proceeds towards ovulation, the dominant follicle shows an increase in β_A-subunit expression, resulting in the increase in inhibin A during the latter part of the follicular phase and reaching a peak at mid-cycle; this in turn decreases FSH leading to suppression of β_B expression in small follicles (Groome *et al.*, 1994). The LH surge is associated with a decline in expression of all the inhibin subunits and with a re-establishment of α- and β_A-subunit expression following the formation of the corpus luteum.

There are no stage-specific changes in the levels of follistatin during the menstrual cycle (Evans *et al.*, 1998; McConnell *et al.*, 1998), and the levels of activin A show a modest increase from the midluteal phase until degeneration of the corpus luteum in the latter part of the cycle (Muttukrishna *et al.*, 1996). These data, in concert with earlier comments made in this review, strongly suggest that while inhibin acts as a negative feedback regulator of FSH secretion, the roles of activin and follistatin are more likely to exert an effect on FSH secretion through paracrine mechanisms within the pituitary gland. However, these proteins and the inhibins may also exert local effects on ovarian folliculogenesis.

Most of the data concerning the local actions of inhibin, activin and follistatin in the ovary arise from non-human species. The inhibin subunits and follistatin have been localized by immunohistochemistry and in-situ hybridization, and the results in general show a similar pattern. These products are expressed in granulosa cells and luteal cells but not in thecal cells (Woodruff *et al.*, 1988; Schwall *et al.*, 1990; Fraser *et al.*, 1993; Roberts *et al.*, 1994; Tuuri *et al.*, 1996; Sidis *et al.*, 1998). However, the localization to luteal cells is a primate phenomenon in keeping with the secretion of inhibin and free α-subunit by the corpus luteum. The pre-antral follicles express both β-subunits and, as the follicle enlarges, α-subunit and follistatin expression increases in keeping with the demonstrated stimulation of these proteins by FSH (Fraser *et al.*, 1993; Roberts *et al.*, 1993).

The actions of activin result in small follicle growth through the stimulation of proliferation of granulosa cells (Rabinovici *et al.*, 1991; Li *et al.*, 1995; Miro *et al.*, 1995; Miro and Hillier, 1996). Additionally, the activin-based enhancement of FSH receptors on granulosa cells further assists the growth of follicles in response to FSH stimulation during the follicular phase of the menstrual cycle (Hasegawa *et al.*, 1988; Xiao *et al.*, 1992). Further synergy with the actions of FSH occurs through the stimulation of increased aromatase expression by activin, resulting in an increase in estradiol production (Hutchinson *et al.*, 1987; Shukovski and Findlay, 1990; Miro *et al.*, 1991). The inhibition of progesterone secretion by activin, when considered with the other actions described above, probably inhibits or delays luteinization.

Since follistatin expression is low in pre-antral follicles, and follistatin can inhibit aromatase and inhibin secretion as well as stimulating progesterone, it seems likely that the local actions of activin maintain the follicle in an FSH-responsive state, whereas follistatin stimulates luteinization (Xiao *et al.*, 1990; Xiao and Findlay, 1991). Further support for this concept emerges from the observation that activin inhibits androgen production by theca cells, thereby limiting estradiol secretion by the developing follicle—an action opposed by inhibin (Hillier *et al.*, 1991; Smyth *et al.*, 1994). However, the capacity of activin to stimulate both follistatin and inhibin production by granulosa cells in turn results in a limitation of its local actions.

Activin appears to stimulate the growth of small follicles in the immature ovary, while in the adult the activin produced by large pre-ovulatory follicles may suppress the growth of surrounding follicles (Mizunuma *et al.*, 1999). Several studies have suggested that inhibin and activin can modulate the capacity of the oocyte to proceed through meiosis (O *et al.*, 1989; Sidis *et al.*, 1998; Silva and Knight, 1998). The local production of activin and inhibin by the cumulus–oocyte complex is supported by the demonstration that cumulus cells express mRNA for all the inhibin subunits and follistatin (Roberts *et al.*, 1993; Sidis *et al.*, 1998). These studies also showed that the oocyte did not express mRNA for the inhibin subunits but expressed all the activin receptor subtypes (IA, IB, IIA, IIB). In keeping with this distribution, activin A stimulated meiotic maturation of oocytes *in vitro* in several species including humans, and this could be inhibited by follistatin (Sadatsuki *et al.*, 1993; Alak *et al.*, 1996, 1998). In addition, inhibin retarded the meiotic maturation of immature rat oocytes *in vitro* (O *et al.*, 1989). These observations suggest that activin may be a useful agent to aid the in-vitro maturation of oocytes for assisted reproduction programmes. In this context, activin A has been shown to increase the capacity of bovine oocytes to form blastocysts (Silva and Knight, 1998).

FSH control of inhibin secretion from the ovary

While several in-vitro studies have demonstrated that FSH can enhance inhibin secretion by granulosa cells, the proliferative action of FSH on granulosa cells can mask the magnitude of the response. However, the detailed studies of Hall and colleagues in GnRH-deficient women receiving pulsatile GnRH secretion have clearly demonstrated the important role of FSH in inhibin B secretion (Hall *et al.*, 1992; Welt *et al.*, 1997). These authors used the observation that the pulse frequency of GnRH administration could significantly alter the magnitude of the FSH response to explore the importance of FSH on in-vivo inhibin secretion. It was noted that increasing the frequency of pulsatile GnRH from every 4 h in the luteal phase to every 90 min at the time of menses is associated with a greater rise in FSH, as well as a rise in inhibin B not seen with the 4 h pulse frequency of GnRH (Hall *et al.*, 1992; Welt *et al.*, 1997). These authors also demonstrated that the interruption of FSH secretion by the use of a GnRH antagonist early in the follicular phase led to a loss of the dominant follicle and a decline both in inhibin A and B secretion. However, if the GnRH antagonist was administered late in the follicular phase, there was an arrest in development of the dominant follicle, which had the capacity to recover after cessation of the GnRH antagonist with a subsequent restoration of inhibin A and estradiol levels, but not those of inhibin B (Welt *et al.*, 1999).

Further evidence of the relationship between FSH and inhibin A and B secretion can be seen in studies of ageing women. In a comparison of immunoactive inhibin levels in the menstrual cycles of perimenopausal women compared with normal cycling women at an earlier age, it was shown that inhibin levels were lower and were matched by an elevated follicular phase FSH secretion (Lenton *et al.*, 1991). These conclusions have been supported by subsequent studies (Klein *et al.*, 1996b; Welt *et al.*,

1999) using specific assays for inhibin A and B, although it was suggested that inhibin B levels decline earlier than inhibin A (Burger *et al.*, 1998; Welt *et al.*, 1999). In contrast, all of these studies suggested that estradiol levels were relatively well maintained in the perimenopausal state (Lee *et al.*, 1988; MacNaughton *et al.*, 1992). Some studies have suggested that the decline in inhibin B may be associated with an increase in activin A, both of which may contribute to the rise in follicular phase FSH levels of cycling women (Reame *et al.*, 1998).

Pregnancy

The measurements of immunoactive inhibin levels by radioimmunoassay established that the levels of inhibin rose initially following the establishment of pregnancy in women conceiving during a study of their menstrual cycles (Lenton *et al.*, 1991). These studies, and others on women without ovaries who conceived by ovum donation and whose inhibin levels were substantially lower than in normal conception, established that in the initial 4 weeks of pregnancy the principal source of inhibin was the corpus luteum (McLachlan *et al.*, 1987b; Yohkaichiya *et al.*, 1991). Nevertheless, the inhibin levels during early pregnancy in women conceiving by ovum donation did rise slightly, consistent with observations that the placental trophoblast secretes inhibin and that the term placenta contains substantial levels of both immunoactive and bioactive inhibin (McLachlan *et al.*, 1986; Riley *et al.*, 1996).

The levels of immunoactive inhibin measured by the 'Monash' radioimmunoassay rose to a peak at 13–16 weeks of pregnancy, plateaued during the second trimester, and rose again in the third trimester to term (Abe *et al.*, 1990; Yohkaichiya *et al.*, 1991). Later studies, using specific ELISAs which detect inhibin A and B, showed that the circulating first- and second-trimester levels were principally inhibin A with the later third-trimester rise being due to both inhibin A and B (Muttukrishna *et al.*, 1995; Illingworth *et al.*, 1996b; Petraglia *et al.*, 1997; Wallace *et al.*, 1997a).

Detailed studies involving partial isolation and characterization of placental extracts demonstrated the presence of inhibin, follistatin and activin (de Kretser *et al.*, 1994). The elution profiles of immunoactive activin displayed three peaks, thereby suggesting the presence of activin A, B and activin AB. However, other studies could not confirm the presence of these species (Yokoyama *et al.*, 1995).

In keeping with the known placental content of activin A and follistatin, studies in sheep confirmed the presence of bioactive and immunoactive activin and follistatin in amniotic fluid and the placenta (Wongprasartsuk *et al.*, 1994). In the human, the early fetoplacental unit was a source of both inhibin A and activin A (Illingworth *et al.*, 1996b; Muttukrishna *et al.*, 1997). Activin A levels rise during pregnancy, with the most marked rise being during the third trimester (Muttukrishna *et al.*, 1996; Petraglia *et al.*, 1997; O'Connor *et al.*, 1999). It was suggested by one group (Petraglia *et al.*, 1994) that activin A levels in serum rose further during labour and noted no increase in women undergoing Caesarean delivery for non-obstructed labour; however, they observed elevated levels where the indication for intervention was obstructed labour. More recently, others (Schneider-Kolsky *et al.*, 2000) were unable to confirm the rise in activin A associated with labour. The reason for the different results in the two studies is not clear, but may reflect differences in the time of sample collection relative to delivery. Recent studies in pregnant sheep have identified that hypoxia is a profound stimulus to fetal activin A production (Jenkin *et al.*, 2001), which raises the possibility that the patients studied in obstructed labour may have subjected the fetus to hypoxia. It is possible that the rise in activin A in late gestation is related to the increasing contractility of uterine smooth muscle, since binding sites for activin have been found in the myometrium of rats infused with radioactively labelled activin A (Draper *et al.*, 1997).

Immunoactive follistatin levels rise during pregnancy with a particularly significant increase in the third trimester (Wakatsuki *et al.*, 1996; O'Connor *et al.*, 1999). These studies suggest that the progressive increase in follistatin in late gestation binds and renders the activin circulating in maternal serum biologically inactive.

Potential clinical applications of inhibin and activin measurement in pregnancy

Prediction of early pregnancy viability

Several studies have proposed that lower levels of either immunoactive inhibin (Yohkaichiya *et al.*, 1991) or inhibin A (Lockwood *et al.*, 1998; Treetampinich *et al.*, 2000) may be a useful marker of early pregnancy loss. However, as there is some overlap between the normal and abnormal ranges this raises some questions as to the application of these observations to routine use in IVF cycles.

Markers of corpus luteum number and function

Progesterone supplements are often used for corpus luteum support in IVF cycles. As such supplements can obscure the use of progesterone as a marker of corpus luteum function, the measurement of inhibin A can provide an excellent protein marker of luteal function (Treetampinich *et al.*, 2000).

Diagnosis of Down's syndrome

Wallace *et al.* (1995, 1998) have demonstrated that inhibin A levels in maternal serum are elevated in women carrying a child with Down's syndrome, whereas the levels of pro-α_C and inhibin B are not. The levels of these proteins in amniotic fluid were also elevated (Wallace *et al.*, 1997b) and the levels of α and β_A mRNAs expression were also increased in the placentae of Down's syndrome pregnancies (Lambert-Messerlian *et al.*, 1998). These observations added to earlier data obtained using assays directed to the inhibin α-subunit (Van Lith *et al.*, 1992; Spencer *et al.*, 1993; Cuckle *et al.*, 1994) which suggested that inhibin measurements could contribute additional diagnostic specificity to the existing components of the triple test (α-fetoprotein, unconjugated estriol and β-hCG), increasing the predictive value to 77%. In a recent study it was observed that, with the triple test, the detection of Down's syndrome was 84%, with a false-positive rate of 21% (Wenstrom *et al.*, 1997). The addition of inhibin A to the other markers raised the detection rate to 90%, with a decline in the false-positive rate to 11%. Attempts to discern the value of inhibin A measurements in first-trimester screening between 8–14 weeks have resulted in conflicting claims that will require further evaluation (Wallace *et al.*, 1995; Wald *et al.*, 1996).

Prediction and diagnosis of pre-eclampsia

In keeping with the need for markers that would identify women at risk of developing pre-eclampsia, Aquilina *et al.* (1999) noted that the second-trimester inhibin A levels in maternal serum of women who later developed pre-eclampsia were elevated above controls. In this study, the predictive value for the diagnosis of pre-eclampsia was 47%, with a specificity of 91%. The positive predictive value was 24% and the negative predictive value was 97%. Similar observations were noted by others (Cuckle *et al.*, 1998) and have been combined with the measurement of serum β-hCG—another potential predictive marker of pre-eclampsia (Muller *et al.*, 1996)—as a predictive test for pre-eclampsia (Lambert-Messerlian *et al.*, 2000). This and other studies suggested that the addition of β-hCG did not improve the sensitivity for pre-eclampsia detection (Aquilina *et al.*, 2000). Activin A levels have also been noted to be elevated in hypertensive women during pregnancy, and some investigators suggest that they are predictive of the subsequent development of pre-eclampsia (Petraglia *et al.*, 1995; Silver *et al.*, 1999). The use of activin A levels in screening for pre-eclampsia appears to provide better predictive value than inhibin A levels (Muttukrishna *et al.*, 2000). Further studies will be necessary to establish the cost-effectiveness of these assays in the early detection of pre-eclampsia.

Diagnosis and follow-up of patients with hydatidiform mole

Application of the 'Monash' radioimmunoassay to the serum of women with hydatidiform mole revealed that immunoactive inhibin levels were elevated in concert with β-hCG (Yohkaichiya *et al.*, 1989). These authors noted that following evacuation of the molar pregnancy, the levels of inhibin declined more rapidly than β-hCG in those women without a continuing trophoblastic source, suggesting that inhibin measurements may provide an earlier prediction of patients requiring further treatment. While more recent studies using either an α-subunit-directed assay or those specific for inhibin A and B confirmed that inhibin was elevated in women with molar pregnancies, the absolute level was not prognostic of outcome, and inhibin was not found to useful as a follow-up marker (Badonnel *et al.*, 1994; Pautier *et al.*, 2001).

Diagnosis of ovarian cancer

The initial observation using the 'Monash' radioimmunoassay indicated that immunoactive inhibin levels were elevated in women with granulosa cell tumours and could predict recurrence (Lappohn *et al.*, 1989). Subsequent studies have shown that 100% of granulosa cell tumours have elevated inhibin levels as assessed by radioimmunoassay and inhibin B ELISA, with the majority also showing increased levels of inhibin A and pro-α_C (Robertson *et al.*, 1999). These studies were followed by others which suggested that the majority of women with mucinous epithelial ovarian tumours had elevated readings in the assay which detects all forms of α-subunit secreted (Burger, 1993; Healy *et al.*, 1993).

In a detailed comparison of the 'Monash' radioimmunoassay with specific assays for inhibin A and B and a pro-α_C assay, Robertson *et al.* (1999) showed that mucinous tumours were detected in 70% of cases by radioimmunoassay and 60% by inhibin B ELISA, whereas serous tumours showed elevated levels in 35% by radioimmunoassay but in <15% with other assays. Using a new α_C-directed immunofluorometric assay (α_C-IFMA), Robertson *et al.* (1999) showed that when this assay was combined with the measurement of CA 125 a very valuable screening test for ovarian tumours was available. This combination of assays showed that in serous tumours, CA 125 was elevated in 94% and the α_C-IFMA in 44%, whereas in mucinous tumours CA 125 was increased in 65% and the α_C-IFMA in 100%. The latter also detected 100% of all granulosa cell tumours. More details of these and other studies are available in a recently published comprehensive review (Risbridger *et al.*, 2001).

Conclusions

This review has focused on the physiology and clinical applications of the inhibins, activins and follistatin in reproductive biology and medicine. It should be remembered that these proteins—particularly the activins and follistatins—have wider roles based on their distribution and actions in many organ systems. These include developmental biology, liver, renal and bone biology, haematopoiesis and inflammation (Phillips, 2001). The implications of these actions are only just emerging and will stimulate rapid expansion of our knowledge that is limited only by the availability of these proteins for experimentation and specific assays for their measurement.

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